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Actions of Ethanol on Voltage-Sensitive Sodium Channels: Effects on Neurotoxin Binding[1]

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ABSTRACT

Exposure of rat forebrain synaptosomes (P_2) to ethanol *in vitro* reduced the specific binding of [^{3}H]batrachotoxinin A 20-α-benzoate ([^{3}H]BTX-B) to voltage-sensitive sodium channels. This effect of ethanol was concentration-dependent and was affected by the membrane potential. Under depolarizing conditions ethanol was significantly more potent at inhibiting [^{3}H]BTX-B binding. Scatchard analysis of [^{3}H]BTX-B binding revealed that ethanol increased the equilibrium binding constant without affecting the apparent maximum number of binding sites. The rate of formation of the [^{3}H]BTX-B/receptor complex was unchanged in the presence of ethanol whereas the rate of dissociation was accelerated by ethanol. These findings are consistent with an indirect allosteric mechanism for inhibition of [^{3}H]BTX-B binding. The binding of [^{3}H]saxitoxin was unaffected by ethanol suggesting that the specific receptor sites in the channel display differential sensitivity to the inhibitory effect of ethanol. These data, in conjunction with ion flux measurements, provide further evidence that ethanol can affect the voltage-sensitive sodium channels in neuronal membranes.

The molecular mechanisms underlying the pharmacological actions of ethanol are at present unknown. There is, however, an abundance of experimental evidence which indicates that intoxicant-anesthetic agents alter the physical properties of biological membranes (Goldstein, 1984). In particular, ethanol has been shown to disorder the lipid portions of intact synaptic membranes (Chin and Goldstein, 1977a; Harris and Schroeder, 1981, 1982), resulting in an increase in membrane fluidity. In this regard, the term "membrane fluidity" is used to describe the degree of mobility of various membrane components (Goldstein, 1984).

The suggestion that intoxication and membrane disorder are causally related is supported by numerous studies utilizing various experimental approaches. Lyon *et al.* (1981) reported a strong correlation between the hypnotic potencies of aliphatic alcohols and their potencies for disordering neuronal synaptosomal plasma membranes. Brain membranes derived from ethanol-tolerant mice are also resistant to the fluidizing effect of ethanol *in vitro* (Chin and Goldstein, 1977b; Harris *et al.*, 1984). In addition, differential sensitivity to the disordering effect of ethanol may occur in membranes derived from mice that have been bred selectively for differential sensitivity to the hypnotic effect of ethanol (Goldstein *et al.*, 1982; Perlman and Goldstein, 1984). However, it has not yet been demonstrated that membrane disordering *per se* causes any known behavioral effect. It is likely that identification of specific membrane functions that are sensitive to changes in membrane order will be useful in the formulation of a mechanism that explains intoxication and/or anesthesia.

A number of studies have examined the effects of ethanol and other intoxicant-anesthetic agents on the movement of ions across excitable membranes (Hunt, 1985). Ethanol, in concentrations that occur *in vivo* (25–100 mM), has been shown to inhibit the neurotoxin-stimulated influx of sodium ions (Mullin and Hunt, 1984, 1985; Harris and Bruno, 1985a) and the potassium-stimulated influx of calcium (Harris and Hood, 1980; Leslie *et al.*, 1983) ions in brain synaptosomes. In addition, the calcium-dependent efflux of rubidium, a model for the calcium-activated efflux of potassium, has been shown to be increased in the presence of ethanol (Yamamoto and Harris, 1983).

Recently, Harris and Bruno (1985b) have studied the effects of a series of chemically diverse membrane perturbants on the lipid order of synaptic plasma membranes and the sodium and calcium fluxes in mouse brain synaptosomes. The results demonstrated that the degree of inhibition of veratridine-dependent sodium influx was proportional to the degree of lipid disordering, in particular the degree of lipid disordering deep in the membrane. The effects of the drugs on calcium uptake were not clearly related to increased membrane disorder.

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ABBREVIATIONS: BTX-B, batrachotoxin A 20-α-benzoate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; STX, saxitoxin.

Thus, it appears that the inhibitory effect of ethanol on sodium influx is a consequence of the increase in membrane fluidity that occurs in the presence of ethanol. To characterize further the action of ethanol on the voltage-sensitive sodium channel, the present study examined the effect of ethanol on the binding of radiolabeled neurotoxins to specific sites in the sodium channels of synaptosomes in which electrophysiological methods are impractical.

Methods

Animals and chemicals. Male Sprague-Dawley rats (200–300 g) were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and were housed two per cage with free access to water and standard laboratory chow. Chemicals and suppliers were as follows: scorpion (Leiurus quinquestriatus) venom, tetrodotoxin and veratridine from Sigma Chemical Co. (St. Louis, MO); [benzoyl-2,5-^{3}H]BTX-B (51 Ci/mmol) from New England Nuclear (Boston, MA). Batrachotoxin was kindly supplied by Dr. John Daly (National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD); and [^{3}H]STX (9.3 Ci/mmol) was a generous gift from Dr. Stephen Davio (Pathophysiology Division, U.S. Army Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD). All other chemicals were obtained from commercial sources and were of analytical grade.

Preparation of synaptosomes. A crude synaptosomal (P_2) fraction was prepared by a modification of the method of Gray and Whittaker (1962). Rats were decapitated and the forebrains (cerebellum and brainstem discarded) were removed and homogenized in ice-cold 0.32 M sucrose and 5 mM K_2HPO_4, pH 7.4 (10 ml/g wet weight), with 10 strokes of a motor driven Teflon-glass homogenizer. The homogenate was then centrifuged at 1000 × g for 10 min. The resulting supernatant was then centrifuged at 17,000 × g for 60 min. The final pellet was resuspended in the indicated volume of the appropriate buffer as described below.

Measurement of radiolabeled neurotoxin binding. The binding of [^{3}H]BTX-B was determined by a modification of the method of Catterall *et al.* (1981). The P_2 pellet was resuspended in binding medium containing the following (millimolar): choline chloride, 130; HEPES-Tris (pH 7.4) 50; glucose, 5.5; $MgSO_4$, 0.8; and KCl, 5.4. Incubations were carried out in a total volume of 300 μl containing 1 μM tetrodotoxin, 45 μg of scorpion venom, [^{3}H]BTX-B and an aliquot of synaptosomes (150–200 μg of protein) in the absence or presence of the indicated concentration of ethanol. Samples were incubated at 36°C for 30 min. The binding reactions were terminated by diluting the samples with 3 ml of ice-cold wash solution and collecting under vacuum on a glass-fiber filter (Whatman GF/C). The filters were then washed three times with 3 ml of wash solution consisting of (millimolar): choline chloride, 163; HEPES (adjusted to pH 7.4 with Tris base), 5; $CaCl_2$, 1.8; and $MgSO_4$, 0.8. Filters were placed in scintillation vials with 15 ml of scintillation cocktail and the tritium content was measured by liquid scintillation spectroscopy with a counting efficiency of 48%. Nonspecific binding was determined in the presence of 300 μM veratridine. Specific binding was calculated by subtracting nonspecific from total binding values. The addition of tetrodotoxin (to inhibit ion flux) and scorpion venom (to increase ligand affinity) to the assay mixture increases markedly the specific component of binding (Willow and Catterall, 1982).

[^{3}H]STX binding was measured by a modification of the method of Krueger *et al.* (1979). The P_2 pellet was resuspended in ice-cold buffer consisting of (millimolar): NaCl, 145; KCl, 5; $MgCl_2$, 1.4; $CaCl_2$, 1.0; NaH_2PO_4, 1.2; glucose, 10; and Tris-HEPES, 20, pH 7.4 to give a protein concentration of 3 mg/ml. Aliquots (100 μl) of the synaptosomes were incubated with [^{3}H]STX in a total volume of 1 ml. After incubation on ice for 60 min the samples were diluted with 5 ml of ice-cold wash solution (150 mM NaCl in 20 mM Tris-HEPES, pH 7.4) and the mixture was collected on glass-fiber filters (Whatman, GF/F). The filters were washed twice with 5 ml of wash solution and the tritium content was measured by liquid scintillation spectroscopy. Nonspecific binding was determined in the presence of 10 μM tetrodotoxin.

Other methods. Statistical analysis was performed using Student's t test. Multiple comparisons with a control were done by analysis of variance and Dunnett's test (1964). Linear segments on Scatchard plots and kinetic experiments were computed by linear regression. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the protein standard.

Results

The effect of increasing concentrations of ethanol on the specific binding of [^{3}H]BTX-B (10 nM) to rat brain synaptosomes is shown in figure 1. Specific binding of [^{3}H]BTX-B to synaptosomal sodium channels in the absence of ethanol was 320 ± 21 fmol/mg of protein. The nonspecific component of binding was unaffected by the presence of ethanol and was 20 to 25% of total [^{3}H]BTX-B binding. The lowest concentration of ethanol that caused a significant inhibition of [^{3}H]BTX-B binding was 75 mM. Ethanol did not completely inhibit the specific binding of [^{3}H]BTX-B as approximately 35 to 40% of [^{3}H]BTX-B binding was unchanged at an ethanol concentration of 800 mM.

To determine if the effect of ethanol on [^{3}H]BTX-B binding was dependent on membrane potential, binding studies were performed in buffer containing 5 or 135 mM KCl. Under these experimental conditions the membrane potential should be approximately −55 and 0 mV, respectively (Blaustein and Goldring, 1975). As the affinity of the polypeptide toxin present in scorpion venom is markedly potential-dependent (Catterall, 1977), the concentration of scorpion venom was increased 10-fold to 1500 μg/ml to ensure essentially complete receptor occupancy at both membrane potentials (Catterall *et al.*, 1981; Willow and Catterall, 1982). Ethanol was somewhat more potent at inhibiting [^{3}H]BTX-B binding under depolarizing conditions as compared with nondepolarizing conditions, as shown in figure 2.

[^{3}H]BTX-B has been shown to bind to a single class of high-affinity receptor sites in voltage-sensitive sodium channels in neuronal membranes (Catterall *et al.*, 1981; Creveling *et al.*, 1983). The equilibrium binding properties of [^{3}H]BTX-B were

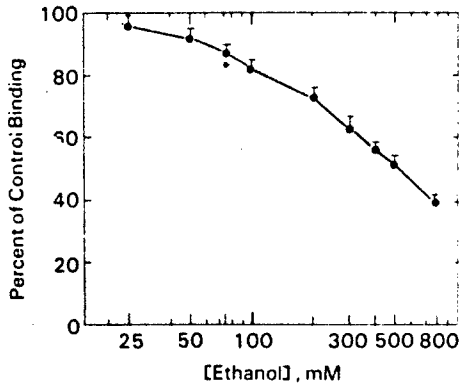

Fig. 1. Concentration-effect curve for inhibition of [^{3}H]BTX-B binding. Duplicate samples of forebrain synaptosomes were incubated with 10 nM [^{3}H]BTX-B (in the presence of scorpion venom, 150 μg/ml) in the presence of increasing concentrations of ethanol for 30 min. Binding was then measured as described under "Methods." The ordinate represents the binding as a percentage of the binding in the absence of ethanol. Values are mean ± S.E.M., n = 4. *Lowest concentration producing a significant ($P < .05$) inhibition of binding.

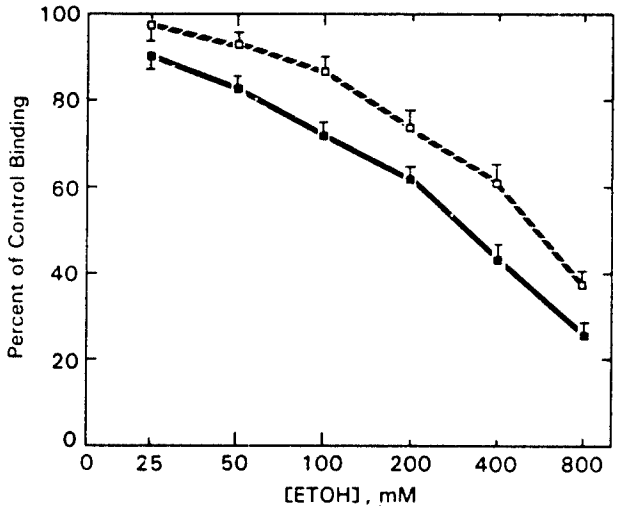

Fig. 2. Effect of ethanol on the binding of [^{3}H]BTX-B under depolarizing and nondepolarizing conditions. Duplicate samples of forebrain synaptosomes were incubated with 10 nM [^{3}H]BTX-B (in the presence of scorpion venom, 1500 μg/ml) and increasing concentrations of ethanol in medium containing 5 mM KCl (□) or 135 mM KCl (■) for 30 min. Binding was measured as described under "Methods." The ordinate represents the binding as a percentage of the binding in the absence of ethanol. Values are the mean ± S.E.M., $n = 3$. The curves are significantly different ($F = 13.54$, dF = 1,4; $p < .025$) by analysis of variance for repeated measures.

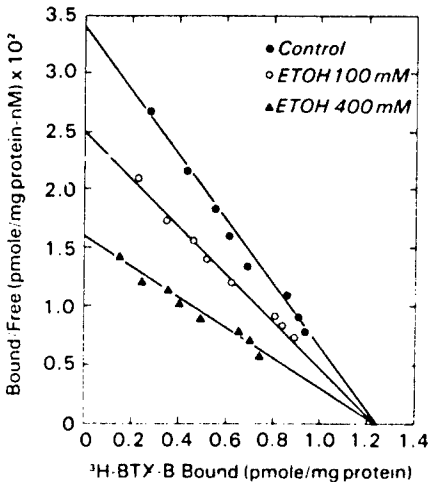

Fig. 3. Scatchard analysis of [^{3}H]BTX-B binding. Duplicate samples of forebrain synaptosomes were incubated with increasing concentrations of [^{3}H]BTX-B under standard assay conditions (scorpion venom concentration was 150 μg/ml) in the presence of the indicated concentration of ethanol for 30 min. Binding was then measured as described under "Methods". Values are the means from four experiments. The S.E.s ranged from 5 to 9% of the mean.

examined over a range of ligand concentrations. Scatchard analysis of data from four experiments yielded a straight line with an apparent maximum number of binding sites equal to 1.24 ± 0.02 pmol/mg of protein. Ethanol, at 100 and 400 mM, increased the K_d from 37.5 ± 3.1 to 50.9 ± 3.2 nM ($P < .05$) and 85.8 ± 6.0 nM ($P < .01$), respectively (fig. 3).

Ethanol (200 mM) did not alter the rate of association of [^{3}H]BTX-B to the binding site on the sodium channel (fig. 4). The dissociation rate constant (k_{-1}) was estimated by incubating samples under standard conditions with [^{3}H]BTX-B (10 nM) for 30 min after which veratridine (300 μM) in the absence or presence of ethanol (400 mM) was added. At various intervals, the samples were filtered and bound [^{3}H]BTX-B was determined. Figure 5 illustrates the accelerated time course of dissociation of [^{3}H]BTX-B from the steady-state complex in the presence of ethanol. Ethanol (400 mM) increased the dissociation rate constant (k_{-1}) from 0.0094 to 0.0169 min^{-1}. These kinetic data indicate that ethanol is an indirect allosteric competitive inhibitor of [^{3}H]BTX-B binding.

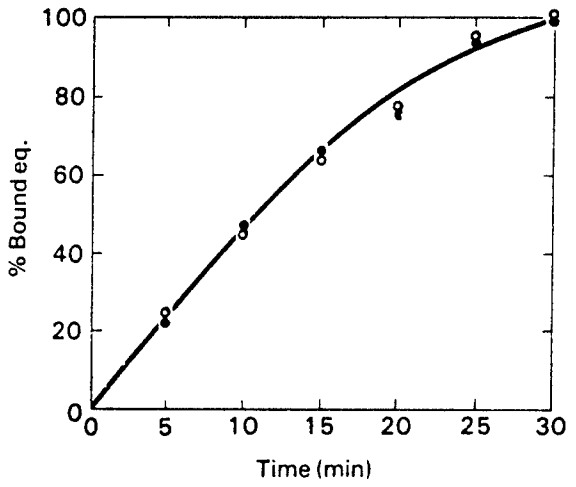

Fig. 4. Time course of formation of the [^{3}H]BTX-B receptor complex. Synaptosomes were incubated for the indicated times with 10 nM [^{3}H]BTX-B (scorpion venom concentration was 150 μg/ml) in the absence (○) and presence (●) of 200 mM ethanol. At each time point, binding was measured as described under "Methods." Values are the means from three experiments. The data are expressed as the percentage of specifically bound [^{3}H]BTX-B at equilibrium (eq) (30 min).

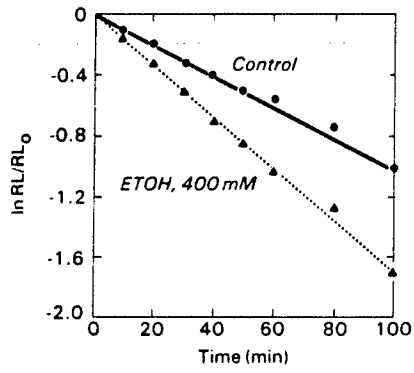

Fig. 5. Effect of ethanol on the dissociation of the [^{3}H]BTX-B/receptor complex. Duplicate samples of forebrain synaptosomes were incubated with 10 nM [^{3}H]BTX-B (scorpion venom concentration was 150 μg/ml) for 30 min. At zero time, veratridine (300 μM) in the absence or presence of ethanol (400 mM) was added and at the indicated times, samples were filtered and binding was measured as described under "Methods." Values are the means from four separate experiments. The standard errors ranged from 4 to 10% of the mean.

Voltage-sensitive sodium channels also contain a receptor for STX and tetrodotoxin which is separate from and does not interact with the receptor for batrachotoxin and veratridine (Catterall, 1980). Results from a Scatchard analysis of the binding of increasing concentrations of [^{3}H]STX in the absence and presence of ethanol are shown in table 1. At a concentration of ethanol which inhibited [^{3}H]BTX-B binding by approxi-

TABLE 1

Scatchard analysis of [^{3}H]STX binding

Values are the means ± S.E.M.; n = number of membrane preparations. Duplicate samples of synaptosomes were incubated on ice for 60 min with increasing concentrations (0.10–10 nM) of [^{3}H]STX in the absence or presence of ethanol *in vitro*.

Group	n	B_{max}*	K_d
		pmol/mg protein	*nM*
Control	4	2.79 ± 0.10	1.45 ± 0.13
Ethanol, 400 mM	4	2.87 ± 0.11	1.53 ± 0.15

* B_{max}, maximum number of binding sites.

mately 40%, there was no effect on [^{3}H]STX binding. These data agree with results from ion flux studies that showed no effect of ethanol on the concentration of tetrodotoxin needed to reduce neurotoxin-stimulated $^{22}Na^+$ influx by 50% (Mullin and Hunt, 1985; Harris and Bruno, 1985a).

Discussion

The present results demonstrate that ethanol inhibits the specific binding of [^{3}H]BTX-B to sodium channels in rat forebrain synaptosomes. Using the intoxication assessment scale of Majchrowicz (1975) and the data in figure 1, ethanol inhibited [^{3}H]BTX-B binding by approximately 9 and 20% at concentrations associated with moderate intoxication (50 mM) and anesthesia (100 mM), respectively. However, under depolarizing conditions in which ethanol is significantly more potent (fig. 2), an intoxicating concentration (50 mM) of ethanol inhibited [^{3}H]BTX-B binding by 19% and an anesthetic concentration (100 mM) caused a 29% reduction in [^{3}H]BTX-B binding. In addition, brain regions differ in sensitivity to the inhibitory effect of ethanol on channel-mediated sodium influx (Harris and Bruno, 1985a). Thus, it may be possible that neuronal sodium channels are involved in some aspects of central nervous system depression associated with intoxication and anesthesia.

Analysis of the effect of ethanol on the equilibrium binding of [^{3}H]BTX-B revealed a concentration-dependent increase in the K_d with no change in the apparent maximum number of binding sites, consistent with a mechanism of competitive inhibition (Tallarida and Jacob, 1979). However, for a number of reasons it is highly unlikely that ethanol interferes with [^{3}H]BTX-B binding by a mechanism of simple competitive inhibition at a single, common site. It is more likely that ethanol inhibits the binding of [^{3}H]BTX-B through an allosteric mechanism. This conclusion is supported by the data from the studies on the kinetic binding properties of [^{3}H]BTX-B. A simple direct competitive inhibitor would be expected to alter the rate of formation of the [^{3}H]BTX-B/receptor complex, whereas ethanol had no effect on this process (fig. 4). In addition, ethanol increased the rate of dissociation of the [^{3}H]BTX-B/receptor complex in the presence of a saturating concentration of veratridine. These findings are compatible with a proposed mechanism of allosteric competitive inhibition.

It is interesting to note that ethanol had no effect on the binding of [^{3}H]STX to rat forebrain synaptosomes. The receptor site for STX is thought to be located in a highly polar, hydrophilic area at the extracellular side of the sodium channel (Narahashi *et al.*, 1966; Angelides and Nutter, 1983, 1984). Thus, the STX receptor site is in an area of the membrane in which the disordering effect of ethanol is rather weak (Chin and Goldstein, 1981; Harris and Schroeder, 1981, 1982). Additionally, although the binding of [^{3}H]STX is inhibited by exposure of the membrane to phospholipases (Baumgold, 1980), the binding of [^{3}H]BTX-B is 10 to 100 times more sensitive to this particular membrane modification (M. J. Mullin, unpublished observation). The binding site for [^{3}H]BTX-B appears to be sensitive to the physical properties of lipid components in the membrane microenvironment.

Recent observations on the covalent binding of a derivative of batrachotoxin suggest that the batrachotoxin binding site is located at the membrane lipid/channel protein interface (Brown, 1985). By disordering lipid domains in the neuronal membrane, ethanol may interfere with specific lipid-protein interactions that are necessary for the binding of batrachotoxin and the subsequent activation of the channel.

A number of drugs that depress neuronal excitability have been shown to inhibit neurotoxin-stimulated sodium uptake and [^{3}H]BTX-B binding (Willow and Catterall, 1982; Creveling *et al.*, 1983). Ethanol at concentrations that inhibit neurotoxin-stimulated sodium uptake also inhibited the binding of [^{3}H]BTX-B, presumably by an indirect allosteric mechanism. However, it is somewhat difficult to directly compare the ion flux and binding assays as there are major differences in the manner in which the assays are performed, including the composition of buffers, the presence or absence of scorpion venom and tetrodotoxin and the time scale of measurement. For example, in the ion flux assays, ethanol reduced the maximal effect of batrachotoxin (Harris and Bruno, 1985a; Mullin and Hunt, 1985) whereas in the binding studies ethanol decreased the affinity of BTX-B for the binding site.

Changes in the physical properties of neuronal membranes due to the presence of ethanol can alter the function of sodium channels which in conjunction with other altered processes may be involved in the depressant effect of ethanol.

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